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13. ABSTRACT *(Maximum 200 words)*

This project focuses on angiogenesis, the growth of new blood capillaries, as a potential target for breast cancer therapy and diagnosis. Our approach was based on our finding that specialized cytoskeletal structures that form at sites of cell-extracellular matrix adhesion, known as the focal adhesion complexes (FACs), contain many of the signal transducing molecules that mediate the capillary growth-stimulating effects of angiogenic mitogens and extracellular matrix. During the last year, we have developed, screened, recloned, and begun to characterize monoclonal antibodies that were generated using isolated FACs as an immunogen. Three different hybridoma fusions have been carried out. These resulted in production of 385 different hybridoma lines of which 26% exhibited positive ELISA and immunofluorescence staining for FAC or cytoskeletal-associated proteins. Eight of these lines have been subcloned at least two times and three of these are currently being used in expression cloning to identify and isolate their molecular antigens. Identification of FAC-associated molecules in capillary cells should facilitate development of new angiogenesis inhibitors as well as alternative methods for breast cancer diagnosis.

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INTRODUCTION

This project is based on the concept that human breast cancer growth may be suppressed by inhibiting tumor angiogenesis -- the growth of capillary blood vessels that feed the tumor -- rather than by targeting the tumor cells themselves. This concept is based on the observation that the cells of solid tumors require continual capillary ingrowth for their own growth and expansion (1). Importantly, while capillary endothelial (CE) cells in tumor microvessels grow very rapidly, normal CE cells are usually quiescent. This differential in CE cell turnover rates therefore provides a potential therapeutic window to selectively prevent tumor expansion without producing generalized cytotoxicity.

Our experimental design is based on past work from our laboratory which revealed that many of the signal transducing molecules that mediate the CE cell growth activation are immobilized on the cytosketon at the site of integrin receptor binding within a specialized adhesion structure that is known as the focal adhesion complex (FAC) (2). In addition to integrating signals elicited by binding of growth factors and extracellular matrix molecules, the FAC also provides a path for transfer of mechanical forces across the cell membrane and to the cytoskeleton (3). Mechanical forces transferred across integrins in the FAC drive changes in cytoskeletal organization and cell shape that are required for cell cycle progression (4,5). In past grant periods, we developed a method to physically isolate intact FACs away from the remainder of the cell and cytoskeleton, and have used this method to demonstrate that isolated FACs retain multiple signal transducing activities in vitro (e.g., protein tyrosine and inositol lipid kinase activities; 2). Work by other groups has similarly identified the FAC as a critical site for signal transduction by matrix and growth factors in other cell types (6)

The GENERAL GOAL of this project was to identify putative molecular targets which could mediate growth modulation by soluble mitogens or angiogenesis inhibitors in CE cells, by focusing on the molecular framework of the FAC. The more specific objective was to develop monoclonal antibodies directed against FAC proteins in order to isolate, sequence, and clone these potential regulatory molecules. Once identified, we would be in a position to determine their role in capillary growth control and thus, to establish a more rational basis for drug design in the field of angiogenesis inhibition. Production of antibodies that recognize growth-associated antigens in CE cells also might form the basis for a more quantitative diagnostic assay for use with tumor biopsy materials.

The specific TASKS proposed under the STATEMENT OF WORK of this grant were:

1. To develop monoclonal antibodies against FAC molecules that are preferentially expressed in growth-stimulated CE cells.

2. To identify antibodies that recognize FAC proteins whose phosphorylation state appears to change in response to treatment with angiogenesis inhibitors.

3. To construct human breast CE cell λgt11 cDNA expression libraries and screen them with these monoclonal antibodies to isolate cDNA clones for the relevant FAC proteins.

4. To explore whether these monoclonal antibodies preferentially detect angiogenic microdomains in histological sections of human breast cancers.

BODY OF THE REPORT

TASK 1:

The major aim of this task was to develop monoclonal antibodies against FAC-related proteins from growth-stimulated CE cells. In the last annual report, we reported preliminary results using intact FACs as a source of antigens for monoclonal antibody preparation. The FACs were isolated from growth factor-stimulated CE cells using RGD-coated magnetic microbeads, as previously described (2). We also described a solid-phase ELISA screening assay that involved use of whole cytoskeletons prepared by extracting adherent cultured cells with detergent. We also developed a novel screening system based on use of microcontact printing to created extracellular matrix-coated adhesive islands in distinct shapes, sizes, and positions on the micron scale (e.g., 5 um wide circles separated by 10 um non-adhesive regions). This ability to control the size and position of FACs in living cells was important because these complexes usually are difficult to identify at low magnification. This problem plus the large variability in FAC size, shape and distribution in standard cultured cells would normally make it difficult to use FAC staining as a high throughput screening method. In last year's report, we demonstrated that CE cells form large FACs along the circumference of 5 um wide adhesive islands which stain positively for known cytoskeletal FAC proteins (e.g., vinculin, talin, paxillin, FAK kinase, phosphotyrosine). Over the past year we have further characterized these structures and confirmed that they contain integrins $\alpha V\beta 3$ and $\alpha 5\beta 1$ (Fig. 1) in a similar staining pattern. In additional studies, we have begun to characterize how different soluble factors (e.g., thrombin, growth factors) alter the levels and distributions of these different FAC components. Interestingly, we have found that the normally circumferential pattern can be increased so that it evenly covers the entire 5 um island when treated with thrombin. The significance of this finding is currently being explored. Most importantly, we have confirmed that the regularity and reproducibility of the staining method makes it well suited for screening monoclonal antibodies in a rapid manner.

In the last annual report, we reported identifying our first set of promising hybridomas that exhibited FAC- and cytoskeleton-specific staining patterns. At the time the report was submitted, we were setting up to clone the most interesting of these cell lines by limiting dilution. The positive hybridomas from the first fusion secreted mainly IgMs. After cloning, only one retained its original staining pattern. The supernatants of these hybridomas also recognized many nonspecific bands by Western blot analysis. For these reasons, we have scaled up our hybridoma production and characterization capabilities over the past year. We produced two different hybridoma fusions using mice that were repeatly immunized, and sera titers were monitored for positive FAC staining using micropatterned substrates as described in the last report. Mice used in the second and third (F2 and F3) fusions were boosted with intact FAC and CSK preparations, respectively. Both fusions were originally screened against cytoskeletal preparations isolated from cells grown in the presence or absence of growth factor (bFGF) using the ELISA method.

As a result of extensive screening of 385 different hybridoma cell lines, we have generated 101 lines (26%) which exhibit preferential FAC or cytoskeletal staining patterns by both ELISA and immunofluorescence microscopy. Unfortunately, none of the positive hybridomas exhibited specific staining in growth factor-stimulated cells. Nevertheless, we chose eight hybridoma lines for future selection and subcloning based on their interesting morphological distribution. Three out of the eight cloned hybridomas maintained the same pattern of immunostaining after two rounds of cloning by dilution; these are called, F2/1A10, F2/1C2 and F3/4A2 (Fig. 2). They secreted antibodies which belong to subclasses IgG1/λ, IgG1/κ, and IgM/κ, respectively, as determined by an isotyping kit. Monoclonal antibody (Mab) F2/1A10 preferentially localizes to FACs in a bright circumferential pattern (Fig. 3) similar to that exhibited by known FAC proteins, such as integrins (Fig. 1). MAb F2/1C2 exhibited a particularly interesting staining pattern in that it stained both FACs and nuclei in most cells (Fig. 3). Mab F3/4A2 differed from integrins and other known FAC proteins in that it labelled both FACs and other cytoskeletal structures. Furthermore, it

preferentially labelled the FACs that formed along the periphery of the spread cell and not the more central adhesion sites (Fig. 3). These novel staining patterns are consistent with the possibility that these Mabs recognize novel FAC proteins. Western blot analysis under reducing conditions revealed that MAbs F2/1A10, F2/1C2 and F3/4A2 recognized SDS-denatured proteins with apparent molecular weights of 200, 40 and 50k Da (Fig. 4). Further cloning steps need to be done with F3/4A2 since the western blot also shows two other bands; this is currently underway.

TASK 2:

This task awaits the development of fully characterized Mabs to FAC proteins. However, we have continued to follow up our past preliminary findings that the angiogenesis inhibitor, TNP-470, alters the phosphorylation state of the FAC signaling protein, FAK kinase. Unfortunately, we have found that these results are not consistent enough to be interpretable at this time. However, we are confident that TNP-470 does alter the structural stiffness of the FAC, as determined by cell magnetometry (3). Thus, we have ceased to pursue the FAK kinase story and will reinitiate this investigation with our new Mabs once they become available.

TASK 3:

In the original proposal, we proposed to construct a human breast CE cell λgt11 cDNA expression library to clone novel FAC proteins that are recognized by our MAbs. Since the time the grant was originally submitted, a recombinant cDNA library has been constructed for the bovine CE cells we used to isolate FAC protein from using a bacteriophage λZAP expression vector. The λZAP II insertion vector is a hybrid vector that combines the high efficiency of λ cloning with the convenience of a plasmid system. It can accomodate inserts from 0-10kb in length and recombinants can be screened with antibody probes. The inserted sequence is expressed as part of a fusion protein after bacteriophage infection of E. coli cells.This library has an average fragment length of 1.6kb and it has successfully used in our department for cloning of the full length MMP-1 gene from our bovine CE cells. The Mabs, F2/1A10, F2/1C2 and F3/4A2, which recognized the denatured form of FAC-associated proteins in Western blots have been selected as the first candidates for expression cloning. These studies were not completed by the time the grant period ended, but are currently underway.

TASK 4:

No new progress.

CONCLUSIONS

The main objective of this grant was to identify specific molecular components of the FAC that mediate CE cell growth stimulation or angiogenesis inhibition. We have developed, subcloned, isolated, and characterized monoclonal antibodies directed against FAC proteins. They were grouped based on their patterns of immunostaining. The subcellular distribution of the molecules recognized by these antibodies was different from previously defined FAC proteins, suggesting that they may represent novel cytoskeletal components. In the future, we hope to use these new antibody reagents we have developed to determine the molecular identity of these cytoskeletal proteins, to analyze their role in angiogenic regulation, and to explore whether they may have utility in breast cancer diagnosis.

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FIGURES

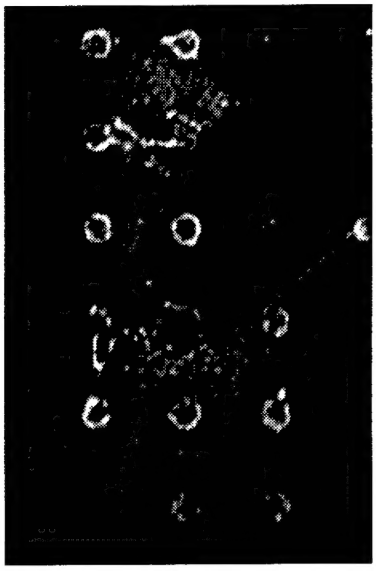

FIG 1. Immunofluorescence micrograph of CE cells adherent to a micropatterned substrate (5 um circles coated with fibronectin separated by 10 um non-adhesive spaces) and stained with antibodies against integrin $\alpha 5\beta 1$. Note the bright circular patterns which represent integrin staining which clearly delineates the periphery of each circular adhesive island.

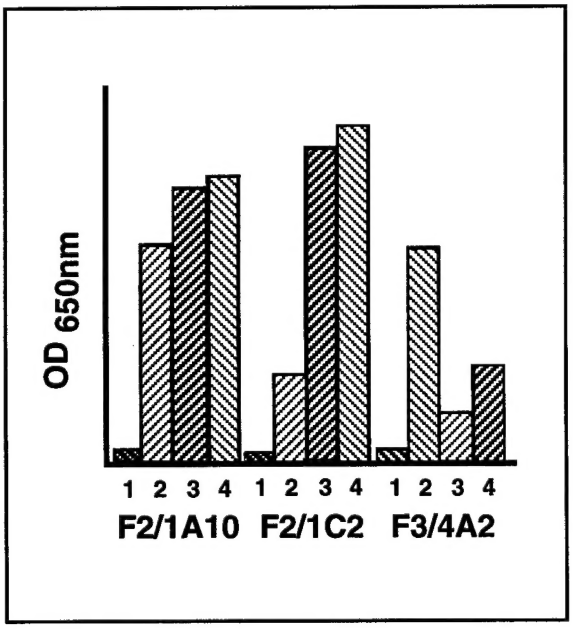

FIG. 2. The histogram shows retention of positive cytoskeletal staining, as detected by solid-phase ELISA, using conditioned medium from hybridoma cell lines F2/1A10, F2/1C2 and F3/4A2 that were selected for further studies. Cytoskeleton-binding activity was measured at 650 nm (OD) in control hybridomas (Bar 1), parental hybridomas (2), and in hybridoma lines after one (3) or two (4) additional rounds of cloning. Note that the cytoskeleton binding activity of the hybridomas was maintained after repeated selections.

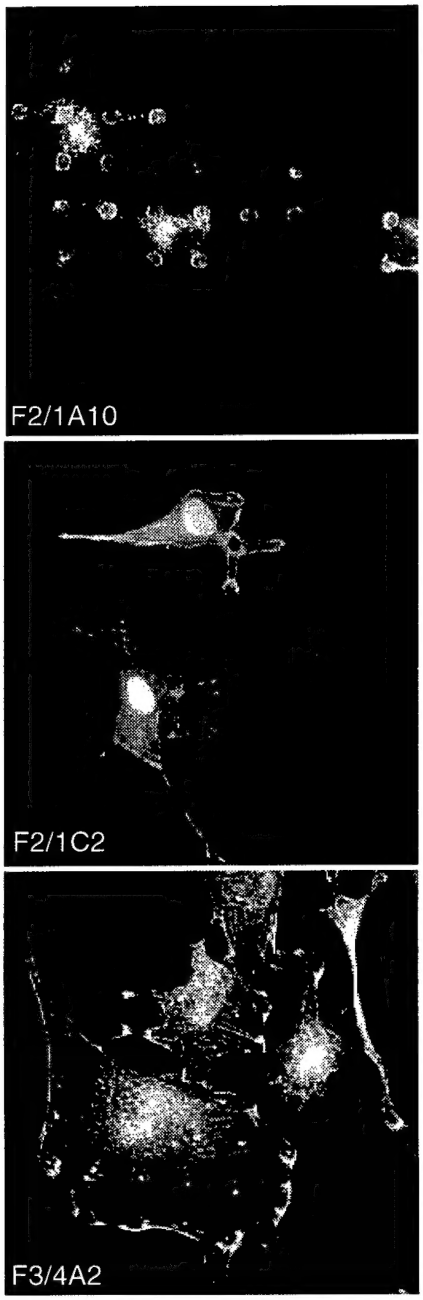

FIG. 3. Immunofluorescence micrographs of CE cells adherent to the same micropatterned substrates as in Fig. 1 and stained with Mabs F2/1A10, F2/1C2 or F3/4A2. Note that all three Mabs retain FAC staining along the periphery of the circular islands, however, F2/1C2 also exhibits bright nuclear staining while F3/4A2 stains both FACs and linear cytoskeletal structures.

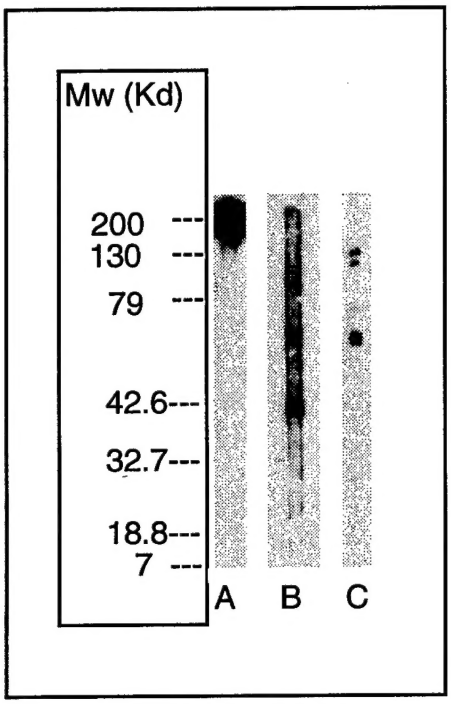

FIG. 4. Western blots of whole CE cell extracts probed with MAbs F2/1A10 (Lane A), F2/1C2 (B) and F3/4A2 (C). Migration of standard molecular weight makers are indicated at the left.